MICROVOLUMETRIC RESPIROMETRY

Methods for Measuring O_2 Consumption and CO_2 Production by Cells and Enzymic Reactions

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INTRODUCTION

When the Arctic Research Laboratory at Point Barrow, Alaska, was ready for use in 1947, it was late autumn. Frost could set in at any time and render unavailable precious material for our projected respiratory studies. This situation brought about an acute need for many more micro respirometers than the dozen we had, and prompted the construction of simple, plastic respirometers drilled in blocks of lucite. Standard specimen vials attached by rubber stoppers were used as respiration and compensating vessels and tuberculin syringes for the measurements of the oxygen consumption (Scholander, 1950). In a few days our Eskimo friends made enough new respirometers to fill our needs. A micro model was similarly constructed and both sizes proved eminently useful on a wide variety of animal and plant material. A model for tissue respiration was designed using standard Warburg vessels. Wennesland (1949) at the same laboratory modified this design to contain an internal rod chamber.

The present respirometers are a further development of the "Barrow respirometers" which are all based on the volumetric principle introduced by Winterstein (1912, 1913). In the volumetric system, the pressure is maintained constant, and the volume changes are read directly. The principle involved can be stated as follows: The pressure of the respiration chamber is balanced against a compensating chamber through a manometer. As gas is absorbed or evolved, it is exactly compensated for by a volumetric device so as to maintain pressure balance with the compensating chamber. The change in gas volume is measured directly on the volumetric device.

The volumetric system offers some conspicuous advantages. The volume changes are read directly as such, and are independent of the gas volume of the apparatus. The only calibrated part is the compensating device (piston

rod). Each respirometer chamber is compensated with a thermobarometer chamber and is, therefore, relatively insensitive to external temperature and pressure changes. The oxygen tension in the respirometer is kept constant during the run. The sensitivity of the respirometer increases with a decreased size of the respiration chamber relative to the compensation chamber and can be adjusted to suit the experiment.

The present method permits the use of inexpensive standard specimen vials as respiration and compensating chambers. The dumping device permits simultaneous dumping in all vials. By means of a micro syringe for volume-compensated injection, gas or liquid can be introduced or withdrawn from the reaction chamber without volume change. By means of this procedure or a simple "lid" technique, RQ. can be followed at intervals in one sample, and the CO_2 content of media, alkali, etc. can easily be determined. The sensitivity and range are comparable to those of the standard Warburg apparatus. A micro model about thirty to forty times more sensitive has also been devised.

I

Macro Manometer and Accessories

The apparatus consists essentially of manometers[1] with vials and accessories for CO_2 absorption, mounting rack for shaking and tilting, micro-syringe for compensated injection, a scale device for reading, and a water bath.

(*a*) *Manometers* (*Fig. 1*).—The manometer consists of a block (*A*, *B*, *C*), and the plug (*D*). There is a hole (rod chamber) in the center of the block, to accommodate the rod (*E*). The upper end of the hole is conical to receive the plug (*D*). The rod chamber is connected to the horizontal respirometer vial (*F*) and with one side of the manometer. The opposite side of the manometer is connected to the vertical compensating vial (thermobarometer) *G*. The vials are attached to the manometer block by means of molded tygon stoppers (shaded in figure) held by lucite buttons. The lucite button for the respirometer vial is drilled to receive the prongs of the absorption plate (*H*). A precision ground rod (*E*), lightly greased, fits through the plug (*D*), and moves at the lower end airtight through a tygon sleeve. When the plug is seated, it seals off the manometer vent to the compensating vial but leaves the manometer vent open to the respirometer vial. When removing the plug, both sides of the manometer are vented simultaneously, guarding against accidental loss of manometer fluid. The position of the rod, *i.e.* the volume displacement, is measured by means of a simple millimeter scale (*K*). A reading refinement consists of a friction-held vernier or cylindrical lens with a hair line, sliding on the millimeter ruler (*L*). The vernier or lens is pushed up on the scale by means of the rod head until the zero end of the scale touches the plug

(*b*) *Carbon Dioxide Absorption.*—CO_2 is absorbed by means of a ½ inch filter paper disc, which fits in a lucite holder (*H*). Another spoon-type of holder permits dumping

[1] The manometers were fabricated by engraving the channels on the face of a lucite block, through a metal jig, with a power engraving tool, and then laminating a face plate of lucite over the engraving.

(*I*). A third type accommodates a glass bucket (Fig. 1, *J*, Fig. 8, *B*), which makes it possible to reclaim the CO_2 trapped by the NaOH (R. Q. determinations, radioactive CO_2, etc.).

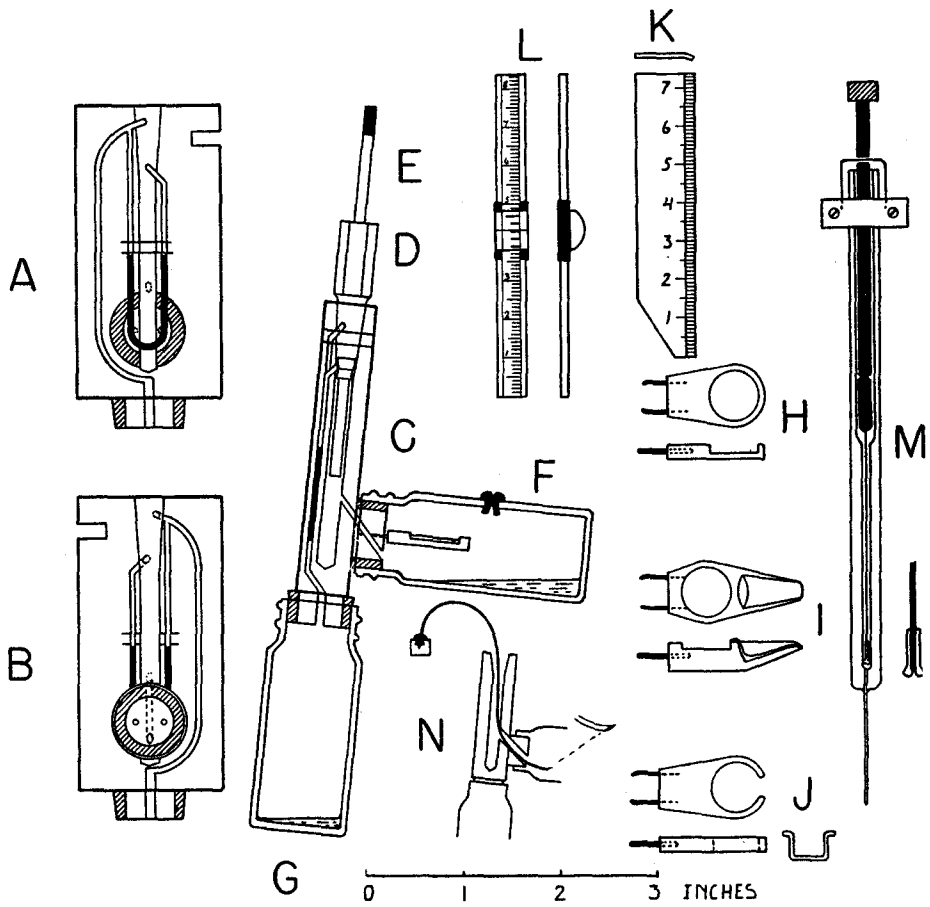

FIG. 1. Macro manometer with accessories. *A*, *B*, *C*, front, back, and side view of manometer. *D*, *E*, plug with rod. *F*, respiration vial with injection port and CO_2 absorption plate (*H*). *G*, compensating vial. *I*, dumping spoon. *J*, holder for glass cup absorber. *K*, simple rod scale. *L*, ruler with sliding hair-line lens. *M*, micro syringe, delivering between stops. *N*, gassing with micro tubing.

(*c*) *Micro Syringe for Volume-Compensated Injection* (*Fig. 1, M*).—This is a device which permits accurate delivery between stops of liquid or gas into the respirometer and withdrawal of precisely the same amount of liquid or gas from the respirometer in one operation. The barrel consists of an upper wider bore tube, and a lower precision bore tube (1.5 mm. I.D.). A blunt hypodermic No. 25 platinum-iridium or stain-

less steel needle, 3 cm. long, is sealed at the end. The coarse upper part of the plunger has two circular grooves carefully spaced. These engage a spring clip set into an adjustable block (Scholander, 1947). The lower part of the plunger consists of a stainless steel wire, with a polyethylene tip, which moves gas and liquid tight in the precision bore tubing. The tip is produced by slipping a 5 mm. length of polyethylene micro tubing over the wire tip, leaving 2 mm. of the tubing protruding. This is rotated near a micro flame, where it shapes into a button.

(*d*) *Vials.*—We have been using standard screw cap vials (Kimble Glass No. 60957). A hole can easily be blown in the side of these to receive a snugly fitting rubber vaccine stopper (*F*). This has a fine slit in the middle, made by means of a No. 20 sharp, greased, hypodermic needle. The slit will serve without leaking for a great many injections by the blunt ended and occasionally greased needle of the micro syringe.

(*e*) *Mounting and Shaking Devices* (*Fig. 2*).—The manometers are mounted on a rack holding 9 lucite swivel blocks (*A*, *B*, *E*). A ball chain under spring tension engages each block adjustably with a spring clip (*B*) so that each block can be properly aligned. A rocker arm (*C*), driven by a connecting rod from a crank device (*D*), activates the swivel blocks with adjustable frequency and amplitude. As the center of oscillation is near the center of the manometer plug (*E*), the manometer rods can be adjusted and read while shaking. The whole rack carrying all the manometers can be tilted around its supporting shaft (*F*) and secured at any angle by handle (*G*). The rack is suspended from the top edge of the water bath, and can be lifted to a support above the water bath when not in use. The shaking rate is normally so adjusted that the liquid washes $\frac{3}{4}$ around the circumference of the vial. This usually requires 220 to 230 R.P.M. and an amplitude of 1 to 1.5 cm. of the bottom of the vial.

(*f*) *Manifold for Gassing* (*Fig. 1 N*).—A square metal tube, holding 9 vaccine plugs is placed across the water bath behind the manometer rack. Hypodermic needles carrying 8 inch lengths of polyethylene micro tubing can be inserted through these ports when necessary. The free ends of the tubes are threaded into the respiration chambers. The excess gas flows out along the tubing. The manifold receives moist gas which has been passed through a bubble flask. If it is necessary to minimize evaporation or distillation during gassing, both bubbler and manifold may be submerged in the water bath.

(*g*) *Water Bath.*—A glass aquarium tank or any tank with a front glass or lucite window may be used. In our instrument, the water bath rests on a low panel box containing the shaking motor, variac, switches, and thermoregulating relay. The stirring motor is mounted independent of the water bath assembly to minimize vibration.

(*h*) *Reagents.*—The manometer fluid consists of one part of boiled kerosene and two parts of nujol. This is amply sensitive and has enough viscosity so that it will not creep in the manometer bore. The fluid is admitted to the manometer opening by means of a syringe with a blunt hypodermic needle curved at the end. Any air bubbles in the oil are easily shaken out.

For carbon dioxide absorption, 10 per cent KOH is generally used. It is essential that the filter paper used in the lucite holders be punched out of coarse paper (Whatman No. 41). Smooth, tight paper gives unsatisfactory absorption. At very high rates

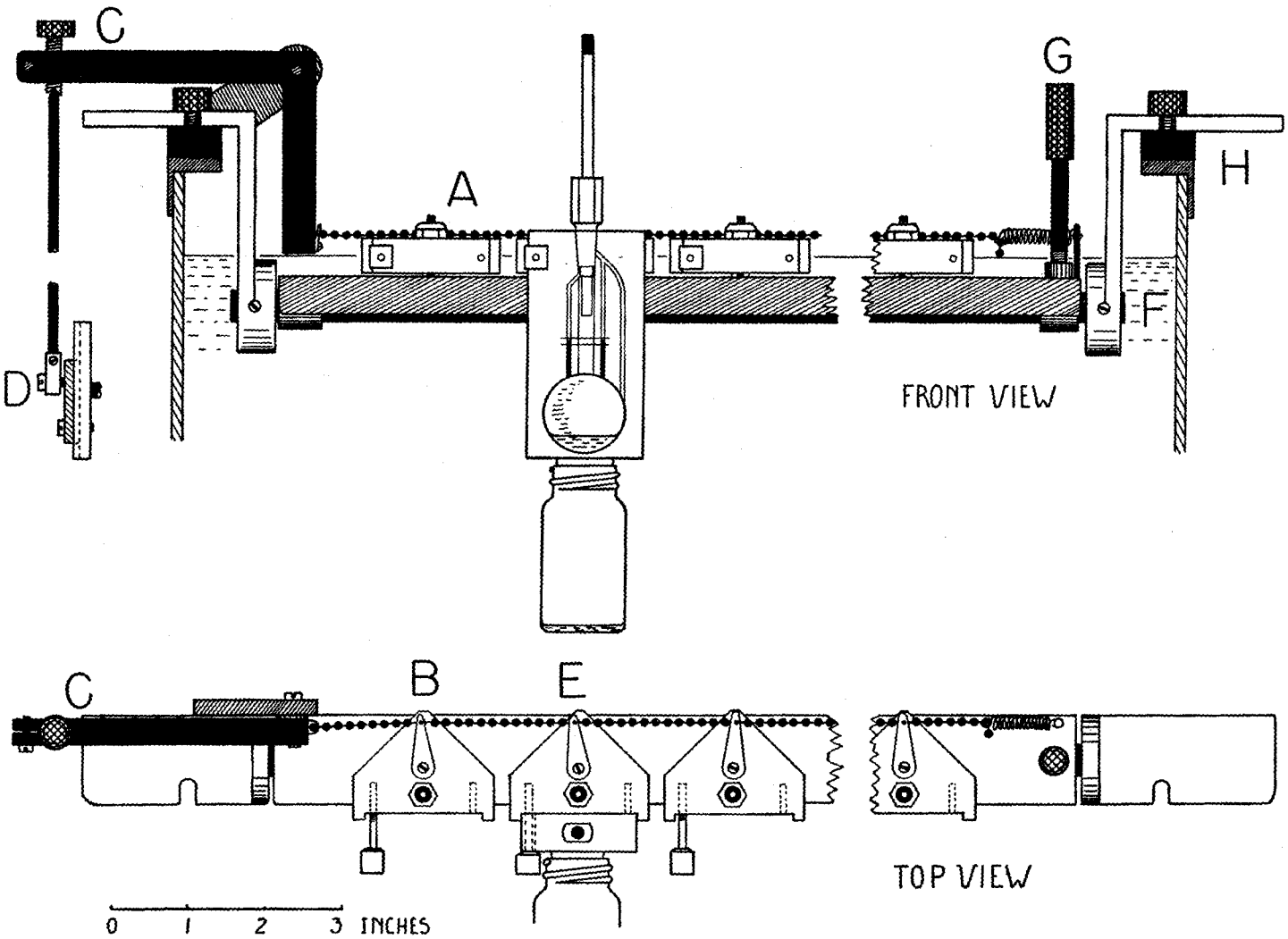

FIG. 2. Mounting and shaking rack for macro and micro manometers. *A*, *B*, *E*, swivel blocks. *C*, rocker arm. *D*, adjustable crank device. *F*, shaft for tilting rack. *G*, lock handle for tilting. *H*, screw attachment to water bath.

of CO_2 production or in runs in which a total of more than $\frac{3}{4}$ of a cc. of CO_2 is produced, it may be necessary to increase the filter paper area and the concentration of the KOH, or to use a glass cup (Fig. 1 *J*) with granular absorber (ascarite) or 20 per cent KOH.

Manometer plugs and rods are lightly greased with heavy nevastane grease (Keystone Co., Philadelphia). Desicote organosilicon compound was used for making the inside surface of the vials hydrophobic when necessary.

II

Calibration

The rods are made of precision ground nylon rod or stainless steel tubing selected to within ±0.5 per cent volume per unit length. The displacement caused by the introduction of grease with the rod through the tygon sleeve is negligible (less than 0.1 per cent unless excessive greasing is used).

The rod factor (converting millimeters rod excursion to cubic millimeter displacement) can be calculated from the diameter of the rod measured by a micrometer, or by injecting water into the respiration vial from the water bath with the micro syringe, and pulling the rod out until the manometer is level. For example, if the micro syringe delivers 298.9 $mm.^3$ by weighing, and the rod after injection must be moved out 36.1 mm. to keep the manometer level, then the rod factor is 298.9:36.1 = 8.28.

The micro syringe is calibrated by weighing the water delivered from the syringe in a small, narrow, weighed vial about 6 × 30 mm. The vial is handled only by forceps so that no temperature gradients develop on the wall. The tall walls and absence of thermal convection will then effectively retard evaporation (Scholander, Edwards, and Irving, 1943), and deliveries within 0.1 mg. can easily be obtained. For deliveries of larger amounts of medium, etc., we have used automatic stop syringes of the clip type (Scholander, 1947).

III

General Techniques

Solutions can be admitted to the medium without disturbing the reading by two methods: (*a*) volume-compensated injection, (*b*) dumping technique.

(*a*) *Volume-Compensated Injection.*—The micro syringe and solution are kept at water bath temperature and usually 100 $mm.^3$ of solution is delivered through the vaccine stopper of the respiration chamber. The needle is introduced until its tip touches the medium, and the fluid is delivered between the stops. The tip is then pulled up above the surface of the medium and the plunger slowly pulled back to the first stop, drawing the same volume of gas into the syringe as the volume of liquid delivered. When the needle is pulled out of the vaccine port, no volume change has been produced by the introduction of the fluid; merely an exchange of a given volume of liquid for an equal volume of gas.

(*b*) *Dumping.*—Instead of injecting, it may sometimes be advantageous to perform conventional dumping. This, unlike injection, can be done simultaneously in all manometers by tilting the rack. For this purpose, a special dumping spoon is used (Fig. 1, *I*). Before use, the dumping spoon is dipped in melted paraffin so that a charge of 150 to 200 mm.^{3} liquid will run off it quantitatively as one large drop when tilted. Alkali solutions and highly hydrophilic protein solutions wet the paraffin coating and cannot be dumped quantitatively in this way, but this method is effective for most aqueous salt or acid solutions. The dumping is effected by tilting the rack quickly and evenly to 45°.

Sometimes a combination of injection and dumping is advantageous. If the injected volume is small, it can be deposited as a drop on the wall and later combined with the medium by dumping. Larger amounts will run into the medium unless separated by a slight ridge pushed into the vial over half its circumference opposite the injection port. The dumping spoon can also be charged by injection.

IV

Calculations

All readings are obtained in terms of millimeters rod excursion which, multiplied by a factor F, gives cubic millimeters of dry gas at 760 mm. pressure and 0°. This requires as known the water bath temperature = T, barometric pressure = P_B, corrected for specific gravity of mercury (= 3 mm. at room temperature) and for water vapor = P_W, and the rod factor converting millimeters rod excursion to cubic millimeters = F_R (in our example, 8.28).

The general formula is mm. rod $\times F$ = mm.^{3} gas consumed or produced, in which

$$F = \frac{P_B - 3 - P_W}{760} \times \frac{273}{T + 273} \times F_R$$

V

Accuracy

The accuracy obtainable by the calibrated apparatus depends upon several factors, namely the volume stability as revealed by blank runs, and the absorption and diffusion characteristics of the gas-liquid system involved.

(*a*) *Determination of Blanks.*—The volume stability of the system as a function of time is determined by blank runs, in which the apparatus is set up with everything present except the test material. Such runs reveal the time for thermoequilibration and any extraneous reason for volume change, such as leaks, oxidations, etc. Properly set up, the system is stable to within the reading error, corresponding to 1 mm.^{3} an hour, and thermoequilibration is complete within 15 minutes if the respirometer is immersed directly into a 37° water bath from room temperature. If preheated, much shorter equilibration time can be obtained.

(*b*) *Oxygen Diffusion Rates.*—The diffusion rate of oxygen into the liquid phase depends largely upon the available surface area and upon the efficiency of the shaking.

For any given rate of oxygen consumption there is the same rate of oxygen diffusion into the medium. This diffusion is maintained by a gradient in oxygen tension pro-

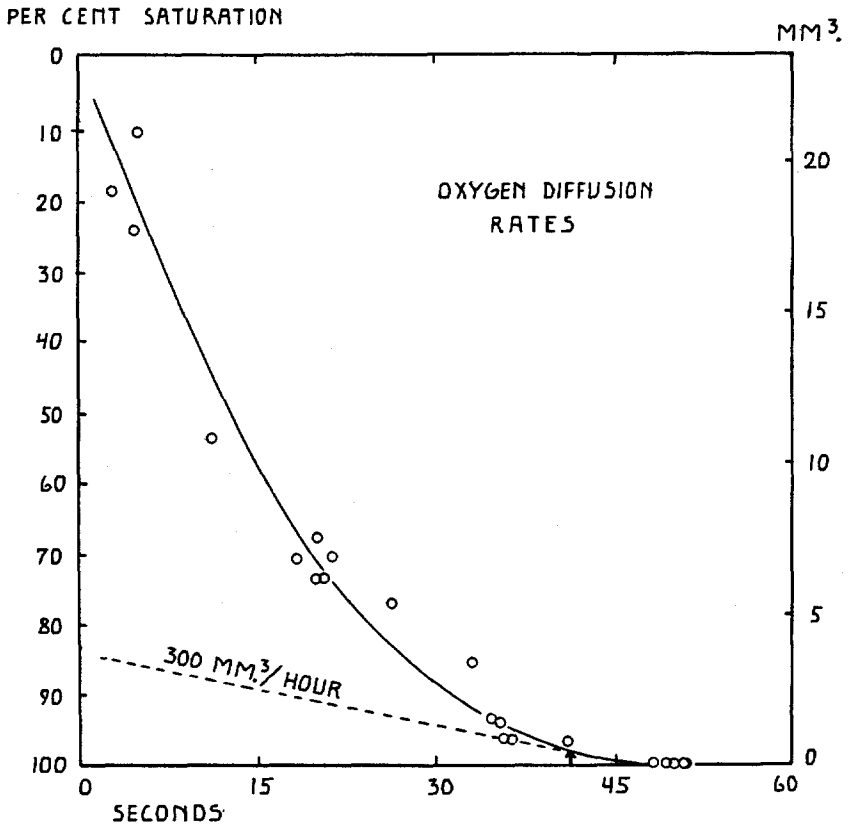

FIG. 3. Diffusion rates of oxygen into 2 cc. gas-free water at 37°, shaking rate 230/min., 27 cc. vial. A normal fast run of 300 mm. oxygen consumption per hour will proceed with only a few per cent desaturation of the medium.

duced by the respiring material. As a result there exists, in the steady state, a certain degree of unsaturation of the medium with respect to oxygen. This may be determined in the following way: The respirometer is gassed through micro tubing from the oxygen manifold. Gas-free water, prepared by boiling or evacuation, is stored in a 10 cc. syringe and kept at water bath temperature. Two cc. of the gas-free water are injected into the respirometer through the micro tubing (Fig. 1, *N*). The plug (*D*) is quickly seated, shaking is started, and the amount of oxygen diffusing into the water is read at short intervals. As the solubility of oxygen in water is known, the saturation at any

given time can be predicted. The data obtained are shown in Fig. 3, in which the degree of oxygen saturation is plotted against time in seconds. The slope of a tangent drawn to this curve gives the rate of oxygen consumption at the point of contact, and the ordinate of this point is the degree of saturation (with oxygen) to be expected. An

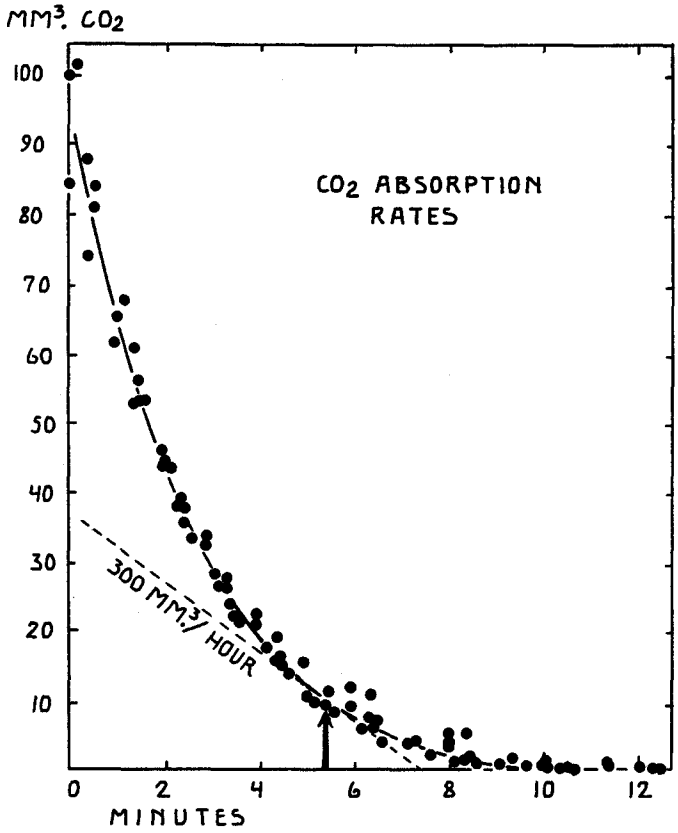

FIG. 4. Absorption rates of CO_2 in 27 cc. vial at 37° using standard ½ inch filter paper disc, with 50 mm.³ 10 per cent KOH. Shaking rate 230/min. 2 cc. water in vial. A normal fast run of 300 mm.³ of CO_2 per hour will have approximately 10 mm.³ CO_2 in transit in the gas phase.

example is given in Fig. 3 in which the rate of oxygen consumption is 300 mm.³ per hour, showing that at this relatively high rate the degree of oxygen unsaturation is less than 3 per cent. In pure oxygen atmosphere, therefore, the diffusion rate through a watery medium is unlikely ever to be a limiting factor.

If runs are made in air, with only ⅕ atmosphere oxygen (maintained by having oxygen in the rod chamber), the situation may become critical, although even here an oxygen consumption of 300 mm.³ per hour will give an oxygen unsaturation of only 10 per cent.

Increasing viscosity will, however, decrease the rate of oxygenation and if the respiring material is allowed to settle to the bottom by insufficient agitation, the free diffusion of oxygen may be seriously hampered and respiration inhibited (see section IX, *c*).

(*c*) *CO_2 Absorption Rates.*—The rate of CO_2 absorption from the gas phase of the respiration chamber depends chiefly upon the area and coarseness of the KOH-wetted filter paper, concentration of KOH, volume of the gas phase, the distances involved, and the agitation. With a properly functioning CO_2 absorber, the rate of CO_2 production equals the rate of CO_2 absorption. The rate of CO_2 absorption with 2 cc. liquid phase and 25 cc. gas phase, using a ½ inch filter paper disc saturated with 10 per cent KOH, is plotted on Fig. 4. The rates were determined by injecting 3 cc. of expired air from a syringe through micro tubing into the respiration chamber, inserting the plug, shaking, and reading. In any system in which CO_2 is being continuously evolved and absorbed, there will always be a certain volume of CO_2 in transit in the gas phase. This amount can be read off from this diagram using the slope of the tangent of the CO_2 absorption curve as a measure of the CO_2 production rate. At a high rate of 300 mm.^{3} CO_2 production per hour only about 10 mm.^{3} of CO_2 was in transit in the 27 cc. gas phase, as is indicated by the arrow in Fig. 4. This equals roughly the CO_2 tension in atmospheric air.

VI

Determination of Oxygen Consumption

(*a*) *Procedure.*—The manometers are filled to the mark with manometer fluid. The compensating chambers, wet on the inside, are slightly moistened around the opening and firmly pressed onto the tygon stoppers. Filter paper discs are placed in the holders (Fig. 1 *H*) which are inserted into the appropriate holes in the manometers (see Fig. 1, *B* and *F*). Fifty mm.^{3} of 10 per cent KOH is added to each filter paper from a tuberculin syringe, and the respiration vials are firmly attached to the stoppers. The manometers are placed on the rack, care being taken that the medium is not tilted back against the stopper. The rack with manometers attached is lowered into the water bath, with the water line as indicated in Fig. 2 and with the rack slightly tilted (Fig. 1, *F*).

If insects or similar material are used, it is necessary to prevent the animals from getting into the alkali absorber, by means of a screen diaphragm. This is a square of plastic fly screening cut diagonal to the weave and pushed into the vial (Scholander, 1950).

If it is desirable to avoid a decrease in oxygen tension when atmospheric air is used in the respiration chamber, the rod chamber must be filled with oxygen. This is accomplished by flushing the rod chamber with O_2 from the O_2 manifold. The lightly greased rod is inserted into the plug, which is lightly greased and firmly seated into the manometer block.

If suspensions are used, the shaker is started and adjusted so that the fluid will oscillate up to 3/4 around the circumference of the vessel. After a period of thermoequilibration (section V, *a*), the rods are adjusted until the manom-

eter reads even. At zero time, the setting is read, and the oxygen consumption is now followed at suitable intervals by resetting and reading. The difference between the readings multiplied by a factor (see section IV) gives the oxygen consumption.

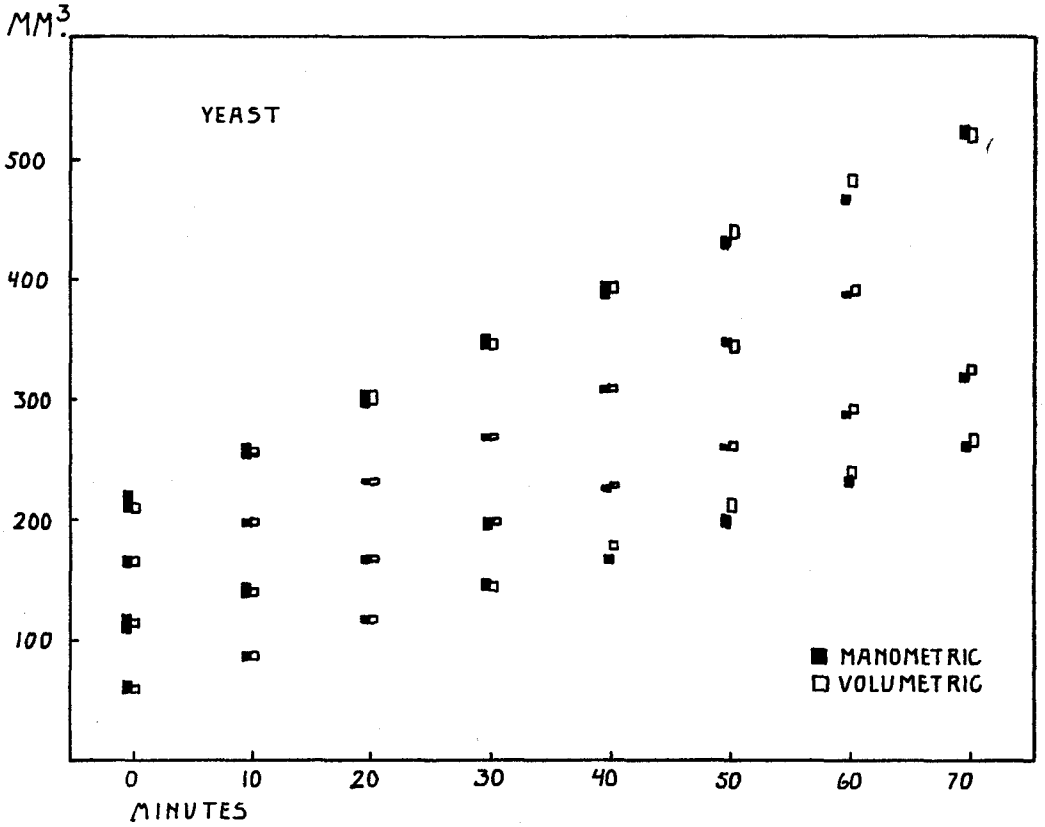

FIG. 5. Oxygen consumption in yeast suspensions run in the Warburg apparatus and in macro manometer. There are 4 separate comparison runs with 5 manometric and 5 volumetric runs in each. Liquid volume, 2 cc., pH 4.6, substrate glucose, temperature 27°. The spread of the data is indicated by the height of the rectangles.

(*b*) *Checks on O_2 Consumption.*—In all conventional manometric or volumetric apparatus, the O_2 consumption is determined as the volume change produced by a respiratory system when CO_2 is simultaneously removed. There is no element in this that specifically determines the O_2 disappearance; hence, the determination depends upon the following physical factors: (1) registration of change in gas volume (or pressure), and (2) the constancy of the amount of CO_2 in the gas phase (in transit between source and absorber). Both of these

elements have been tested separately (see section V). As conceivably other factors may influence the rate of respiration, like the type of agitation, toxicity

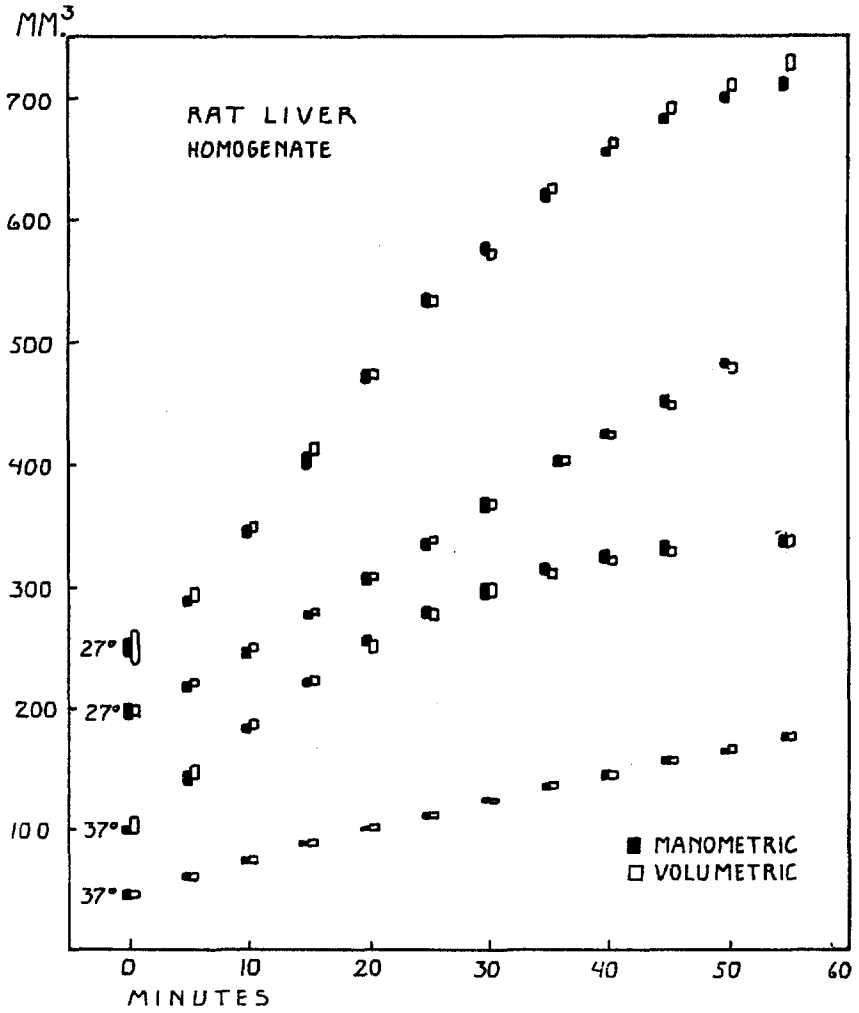

FIG. 6. Oxygen consumption in rat liver homogenate. There are 4 separate double runs with 5 manometric and 5 volumetric determinations in each. Liquid volume 2 cc., pH 7.4, substrate sodium succinate, temperature 37°.

from manometer fluid, grease, plastics involved, etc., we have made comparative runs between the Warburg apparatus and the volumetric machine on yeast (Fig. 5) and liver homogenates (Fig. 6). The runs, whether linear or not, are all practically identical in magnitude of O_2 consumption and spread between individual observations.

VII

CO_2 Determinations

(*a*) *Procedure.*—To determine the amount of CO_2 in a medium or other solution in the respirometer, acid is added to it by volume-compensated injection; or the unknown may be injected into a respirometer containing acid. In each case, the CO_2 evolved equals the observed volume increase plus the

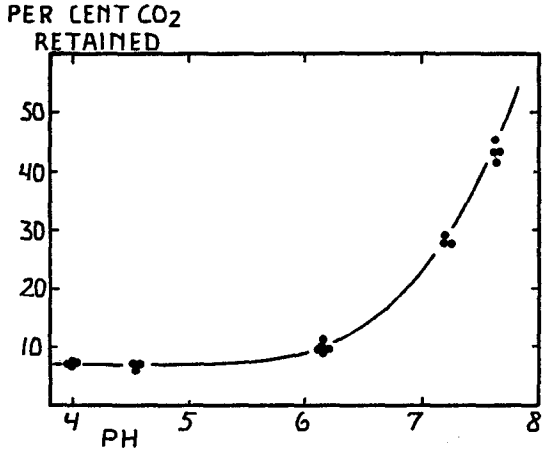

FIG. 7. CO_2 retention in 2 cc. 10 mM buffer solutions of known initial pH, 20 cc. gas phase, determined by introducing 200 mm.^{3} CO_2 by volume-compensated injection.

CO_2 that remains dissolved in the liquid phase. The total CO_2 content is calculated from the observed volume increase according to the formula:

$$V = V_i\left(\frac{V_1 \times \alpha}{V_g} + 1\right)$$

in which V is the total volume of CO_2, V_i is observed increase, V_1 the liquid volume in the respirometer vial, V_g the gas volume of the respirometer vial and the rod chamber, and α is the absorption coefficient of CO_2 in the liquid.

Above pH 4 increasing amounts of CO_2 remain in the solution as bicarbonate. However, no significant error is introduced by figuring CO_2 retention as H_2CO_3 until pH values are in excess of 5.5 (Fig. 7). The CO_2 retention at any pH value may be estimated empirically. Pure CO_2 comparable to the amount evolved is injected into the thermoequilibrated respiration chamber containing the desired medium and the same amount of gas is withdrawn. The manometer is shaken and read. If 300 mm.^{3} were injected and correspond to 36.0 mm. rod excursion and the volume after the injection shrinks 3.6 mm., then the retention is 10 per cent for that case.

For CO_2 determinations in media, plasma, etc., it is usually possible to inject

100 mm.^{3} into 0.5 cc. or less of 2 per cent sulfuric acid in a 27 cc. air space, keeping the pH below 4 and the retention at or below 1 per cent. If a relatively large amount of liquid must be used, the CO_2 retention becomes large and may then preferably be determined empirically as described above, even at a low pH.

(*b*) *Accuracy.*— We have tested the accuracy by injecting known amounts of standard carbonate solution into acid (Table I), and by dumping different

TABLE I

Determinations of CO_2 in Standard Carbonate Solutions by Macro Manometers

98.8 mm.^{3} Na_2CO_3 solution injected into 0.4 cc. 2 per cent sulfuric acid using 27 cc. vial. Values corrected for CO_2 retention in solution = 1 per cent.

Mm.^{3} CO_2 per 100 mm.^{3} solution	Found								Averages*
Mm.^{3} 300	302	300	305	305	298	297	304	299	300.8
Per cent	100.6	100.1	101.6	101.6	99.4	99.2	101.3	99.6	100.3
Mm.^{3} 200	198	197	203	200	202	195	204	200	199.9
Per cent	99.1	98.4	101.4	100.3	101.0	97.7	101.7	99.9	100.0
Mm.^{3} 100	99.1	99.1	100.6	102.1	101.3	101.3	102.8	102.1	100.0
Per cent	99.1	99.1	100.6	102.1	101.3	101.3	102.8	102.1	100.0
Mm.^{3} 50	48.4	49.9	49.9	48.4	48.4	51.4	52.2	51.4	50.0
Per cent	103.2	100.2	100.2	103.2	103.2	97.3	95.8	97.3	100.0

* Calculated from original figures.

TABLE II

Determinations of CO_2 in Urea by Urease by Macro Manometer

101.8 mm.^{3} 0.1 M urea injected into 7 per cent Arlington urease in 0.3 cc. pH 4.4, acetate buffer. Values corrected for CO_2 retention = 1 per cent.

	Theoretical	Found								Averages
Mm.^{3}	228	225	228	228	226	229	229	228	226	227
Per cent		98.7	100.0	99.7	99.7	100.4	100.4	100.0	99.1	99.6

strengths of urea solution into a urease solution (Table II). The results over the whole range are within ±1 to 2 per cent of the theoretical value. This simple and quick procedure has been applied successfully to CO_2 determination in plasma (Kinoshita, Bunker, and Scholander).

VIII

Determination of Oxygen Consumption and CO_2 Production (R. Q.)

Two techniques have been worked out, permitting periodical determinations of oxygen consumption as well as CO_2 production and hence R. Q.; one, using

periodical covering of the CO_2 absorber by a lid; the other, a periodical renewal of the NaOH in the absorption cup and volumetric determination of the amount of CO_2 trapped in it.

(*a*) *The Lid Technique.*—This technique depends upon periodical closing and opening of the absorber. When the absorber is closed, CO_2 accumulates and the volumetric change in this period gives the difference between O_2 consumption and CO_2 production, corrected for the fraction of CO_2 which is dissolved in the liquid phase of the medium and the organism. The smaller this fraction is, the more accuracy can be expected. Hence, the system works well in acid media or at high Q_{O_2} as in acid yeast suspensions, with many insects, etc. Above pH 5.5–6, the method becomes inaccurate due to retention of CO_2 in solution (*cf.* Fig. 7).

The technique (Fig. 8,*A*) requires a circular lucite lid with $\frac{1}{2}$ inch recess for a filter paper disk charged with 10 per cent KOH. The opening is ground

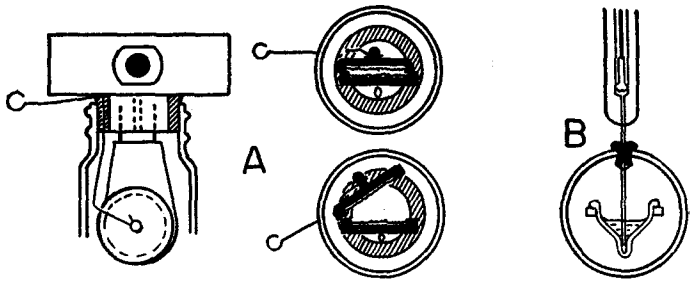

FIG. 8. *A*, lid technique for R.Q. determinations. *B*, alkali exchange technique for R.Q. or other determinations requiring quantitative periodical renewal of the alkali.

flat, and likewise the opening of the regular filter paper holder. A wire passes through the tygon stopper and is bent so that the lid can be opened and closed from the outside by flipping the wire lever. A drop of nujol oil around the edges is necessary to produce an airtight seal when the lid is closed. The respiratory material is introduced into the respiration chamber and run with the lid open for, *e.g.* 10 minutes, closed for 10 minutes, open for 10 minutes, and so on, recording the volume changes. With such a schedule, the R. Q. can be calculated every 20 minutes. Four such records are given in Fig. 9, showing the R. Q. obtained from yeast cells taken directly from the cake and washed several times in water. In the two lower curves, glucose was added, illustrating how changes in R. Q. can be followed by this method. In other experiments, the yeast cells were given acetate as substrate which is metabolized with a known R. Q. of 1 (Fig. 9).

The oxidation of acetate by yeast proceeds *via* the tricarboxylic acid cycle as demonstrated by Lynen (1942). The complete oxidation of this fatty acid would give an R. Q. of 1: $CH_3COOH + 2\ O_2 = 2\ CO_2 + 2\ H_2O$. At the sugges-

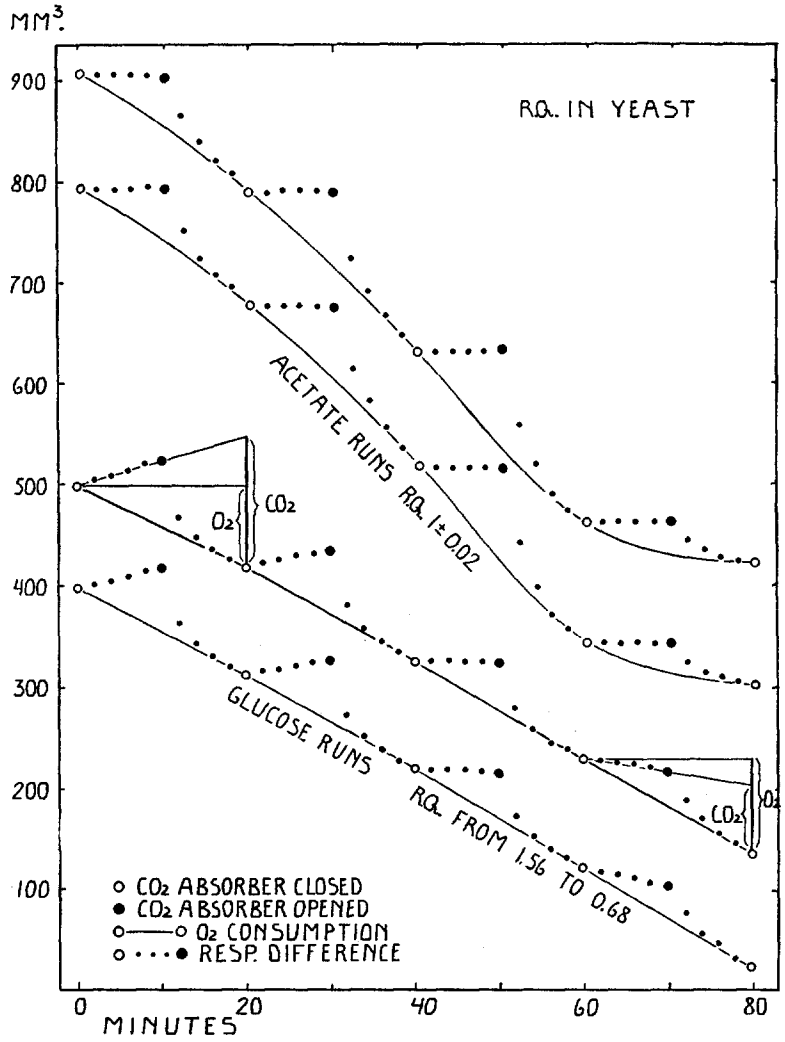

FIG. 9. R.Q. determinations in yeast suspensions with lid technique. 2 upper curves starved yeast in sodium acetate substrate, pH 4.4. 2 lower curves starved yeast with glucose substrate, pH 4.4. The R.Q. changes are as follows:—

1.56	1.26	0.95	0.72	(0.66)
1.48	1.21	0.91	0.68	(0.61)

The construction of the oxygen consumption and the CO_2 production from the curve is indicated in the figure.

tion of Dr. E. S. G. Barron, we used this reaction to check the validity of our method.

(*b*) *Alkali Exchange Technique.*—In this technique, a measured amount of CO_2-free NaOH is periodically renewed in the absorption cup. The CO_2 in the withdrawn NaOH is liberated by acid in another respirometer, and the volume measured.

CO_2-free NaOH is used as absorber and is prepared by drawing 1 cc. saturated clear NaOH solution (approximately 18 N) into a tuberculin syringe. This is injected anaerobically into 6 cc. CO_2-free water held in a vaccine stopper-capped syringe. The mixture is shaken and yields approximately a 10 per cent solution of nearly CO_2-free NaOH. The syringe is kept tip up in a beaker of water, which covers the barrel opening to prevent sticking. The glass absorption cup has a drawn-out bottom (Fig. 8,*B*) which permits exchange of the solution within 1 per cent. The cup is placed directly underneath the injection

TABLE III

R.Q. *Determinations of Yeast in Acetate Solution Using Alkali Transfer Technique*

pH 4.4. Theoretical R.Q. = 1.00.

Time, *min.*	0–10	10–20	20–30	30–40	40–50
Run No. I	1.00	1.02	1.02	1.03	1.04
II	1.00	0.96	1.00		
III	0.98	1.03	1.03	1.00	
IV	0.97	0.98	0.94	0.98	
V	1.03	1.01	1.03	1.02	0.97
VI	1.04	1.02	(1.48)	1.00	1.03

Average 1.00 ± 0.03.

port of the respiration vial. A drop of 2 per cent sulfuric acid is placed in the sinus of the under side of the vaccine stopper to prevent any NaOH from sticking to the port when the injection needle is withdrawn.

After assembly of the manometer, 1 micro syringe full (100 $mm.^3$) of the 10 per cent NaOH is delivered into the absorption cup through the vaccine port. At suitable time intervals, the NaOH is exchanged and the used NaOH is injected into another manometer containing 0.4 cc. 2 per cent H_2SO_4 and the CO_2 evolved is measured. The CO_2 blank value of the NaOH is similarly determined. It is possible to follow by 10 minute intervals O_2 consumption and CO_2 production in a run of normal intensity, giving R. Q. within ± 0.03 unit (Table III).

IX

Micro Manometer

(*a*) *Description and Operation.*—A smaller model, 30 to 40 times as sensitive as the larger, has also been developed (Fig. 10). It is in all essential parts the same as

the larger unit. The reaction chamber may be placed on the side so as to make its contents more visible. As vials are used small cylindrical specimen vials (A. H. Thomas, Philadelphia, Catalogue No. 9805) which fit onto conical buttons by means of a tygon ring cut from a thin walled $\frac{1}{8}$ inch tubing. The rings are pushed up on the conical button until the vial fits snugly over it. The edge of the vial must be pushed completely over the ring. The manometer rod (*A*) is made of a No. 20 hypodermic needle tubing closed at the ends. It is moved by a screw-threaded rod and passes through a small hole in a tygon disc which makes an airtight bearing at the end of the plug. Equal volumes of boiled kerosene and nujol serve as manometer fluid. The manometers fit on adapters which screw onto the swivel blocks for the large manometers. The manometers can be tilted individually or simultaneously.

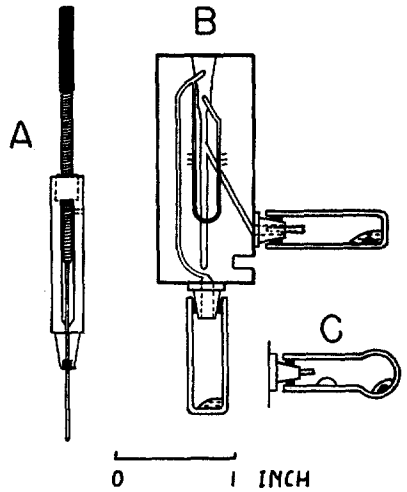

FIG. 10. Micro manometer. *A*, plug with screw feed for micro rod. *B*, manometer block with horizontal respiration vial. *C*, respiration vial loaded for dumping of the drop in the neck into the bulb.

A small rectangular piece of filter paper inserted into the hole of the connecting button and protruding a few millimeters into the vial (*B*) serves as the CO_2 absorber. It is saturated with a drop of 5 per cent KOH. The respiratory material is placed at the bottom of the vial. It is often practical to have the bottom blown out bulb-shaped (*C*) and the inside of the vial made hydrophobic with desicote (National Technical Laboratories).

When dumping is desired, the solution is deposited from a micro syringe as a separate drop on the hydrophobic wall (*C*). It can also be done through a micro tube, after assembly of the apparatus (Fig. 1, *N*). For dumping, the respirometer is tilted and, if necessary, it is given a few rapid oscillations.

The gassing procedure is the same as for the larger unit (Fig. 1, *N*). It is necessary in the micro method to keep evaporation losses to a minimum (see page 378).

The rods used deliver 0.393 $mm.^3$ per mm. movement. For the delivery of desired amounts of fluid into the respiration vial, we have used the micro syringe (Fig. 1, *N*)

with an extra stop delivering 26.4 mm.[3] For other volumes we have used a micrometer burette (Scholander, 1942), with polyethylene micro tubing attached to the tip.

(*b*) *Blanks.*—The volume stability of the micro manometer is within 0.03 mm.[3] per hour. Time for thermoequilibration from room temperature to 37° is 10 to 15 minutes.

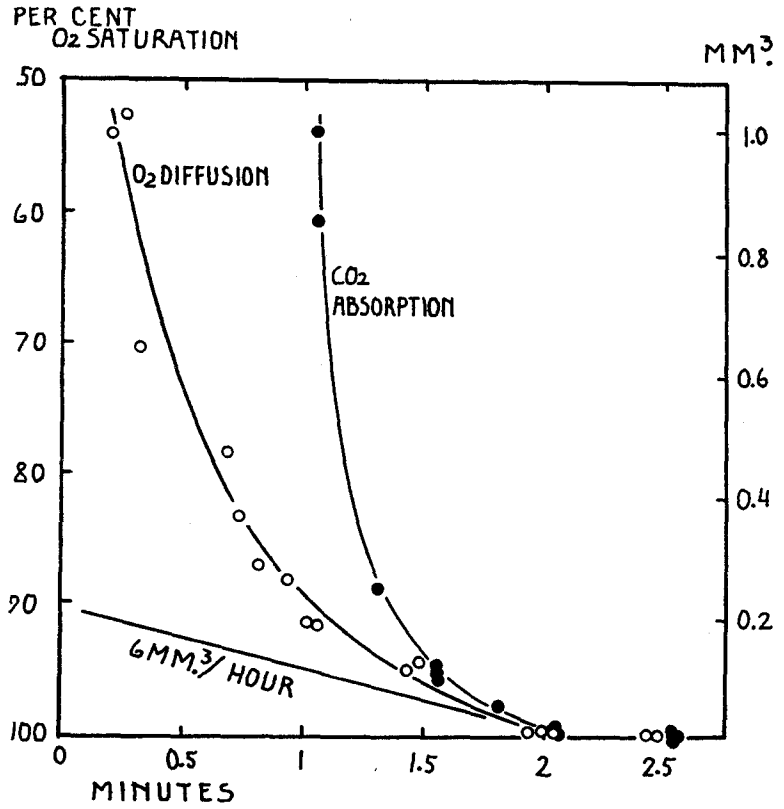

FIG. 11. Oxygen diffusion rates and CO_2 absorption rates in micro manometer. Both are ample to take care of even the most rapid runs.

(*c*) *Oxygen Diffusion Rates.*—The diffusion rate of oxygen into water was determined as described for the larger manometer. After gassing with oxygen, gas-free water was injected through micro tubing and the diffusion of oxygen into the water was registered. In a hydrophilic vial, the resaturation rate was usually too rapid to be measured even without shaking. In hydrophobic vials, the resaturation rate was likewise considerable. At a high rate (6 mm.[3] per hour) of oxygen diffusion, 80 mm.[3] water was less than 5 per cent desaturated (Fig. 11). With a smaller amount of liquid and a lower oxygen consumption, the saturation decreases imperceptibly. With such excellent aeration conditions,

due to the relatively large surface and short diffusion distance through the medium, it was thought that shaking possibly could be omitted. In a run of yeast suspension in glucose, the shaking was stopped for 20 minutes. When the shaking was stopped, the respiration rate dropped in a few minutes to merely 10 per cent of the value obtained while shaking, and the normal respiration rate was resumed when shaking again was started (Fig. 12). This proves the important point that, even though the diffusion rate in the medium may be ample as compared with the respiration rate, any settling of cells against the bottom or on top of each other may effectively block off the diffusion of gases or substrate, so that only the exposed outer layer of cells can respire at normal rate. It seems therefore necessary to provide agitation even in micro

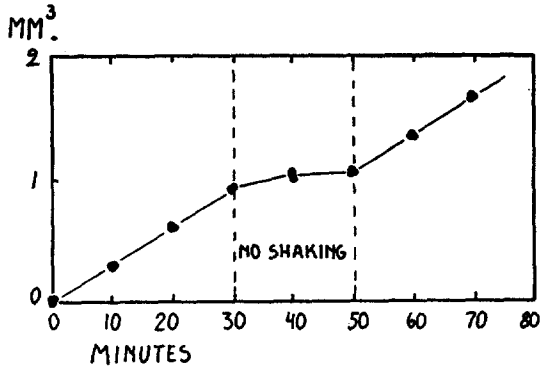

FIG. 12. Effect of shaking on the respiration of a drop of 26 mm.^{3} yeast suspension. When shaking stops, respiration drops to 10 per cent of its normal rate, due to settling of the cells and blocking off of normal access to oxygen and substrate.

methods whenever such situations can arise. It should also be noted that high viscosity will impede equilibration.

(*d*) *CO_2 Absorption Rate.*—This was determined by injecting 100 mm.^{3} expired air into a 1 cc. vial charged with filter paper protruding 2 to 3 mm. and moistened with 5 mm.^{3} 5 per cent KOH. The absorption rate from the gas phase is very rapid (Fig. 11). With the highest practical rate of CO_2 evolution (6 mm.^{3} per hour), the CO_2 in transit is hardly measureable. It would be even smaller in slower runs and smaller vials and is hence never likely to become a limiting factor.

(*e*) *Calculations.*—The formula is the same as for the larger manometer (see section IV). The rod factor in our size is 0.393 mm.^{3} per mm. rod movement.

(*f*) *Checks on Oxygen Consumption.*—Several runs were made on yeast suspensions and liver homogenates, comparing the oxygen consumptions obtained by the macro and micro methods. In sixty-three runs, the results were practically

identical as to absolute values and spread. The amounts of yeast suspension and liver homogenate were about 1 cc. in the macro manometers and about 26 mm.^{3} in the micro manometers.

(*g*) *CO_2 Determinations.*—The accuracy of CO_2 determinations was checked by dumping standard carbonate solution into acid, and by dumping standard urea into urease. The accuracy is satisfactory within 1 to 2 per cent even with determination of only 0.006 mg. of urea or 2.2 mm.^{3} CO_2.

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